

# Rapid Method for Determining the Zein Content of Whole Maize Seed or Isolated Endosperm<sup>1</sup>

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## ABSTRACT

Ground maize seed is extracted with 70% ethanol at 60°C. for 90 min. and an aliquot transferred to a filter paper disc. Discs are washed in bulk through the series: saline, water, ether, acetone, ether, to remove non-zein impurities, and the zein nitrogen is determined by micro-Kjeldahl analysis on the air-dried discs. Reproducibility is good and the technique permits large numbers of samples to be processed quickly and simply.

In studying the developmental patterns of protein synthesis in maize endosperm, the need arose for a rapid, specific method for determining the maize prolamine, zein. Any procedure based on the Osborne (1) fractionation of maize protein using conventional separatory techniques, i.e., repeated extraction and centrifugation, is too time-consuming when large numbers of samples are involved. Craine et al. (2) published a rapid turbidimetric method based on the precipitation of zein from alcoholic solution, but reported some problems relating to reproducibility when the assay was applied to crude ethanol-water extracts of corn. The turbidimetric method has been proposed as a rapid procedure for estimating lysine in maize selections, based on the statistical demonstration of an inverse relationship between lysine and zein (3).

The present method involves the direct extraction of zein from ground endosperm, or whole kernels, with 70% ethanol, precipitation of the zein in the fibrous matrix of a small filter paper disc, removal of non-zein nitrogenous impurities by passing the disc and appropriate control discs through a series of wash solutions, and determination of the total nitrogen of the disc plus zein (or control disc).

The virtue of the method, which has been in use for several years in this laboratory, is that it permits large numbers of zein analyses to be performed rapidly, while retaining the specificity of nitrogen analysis as a base for determining the amount of protein present.

## MATERIALS AND METHODS

### Zein Standard

The procedure of Baudet et al. (4) for determining "cold," "hot," and "total" zein was employed on replicate (ten) samples of the maize inbred W64A. This provided a) an estimate of the variability (coefficient of variation) of the Baudet et al. method for comparison with the method under development, b) a standard maize preparation of known zein content for use in establishing the optimum conditions of zein extraction, and c) a specimen of laboratory zein (from pooling the 10 replicate zein samples) suitable as a standard for investigating disc washing techniques. These various uses are detailed in the text.

### Sample Preparation

Mature whole kernels, or hand-dissected endosperms, are ground directly.

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Immature material, (whole kernels, isolated endosperm) is first lyophilized to constant weight. A minimum of 20 kernels or endosperms (more for immature material) are disintegrated in a Waring Blendor for 1 min. and a small (0.5 g.) sample of this material is ground further in a Wig-L-Bug (Crescent Dental Mfg. Co., Chicago) for 3 min.

#### Assay Procedure

Two replicate 100-mg. samples of finely ground whole kernel or endosperm powder and 0.4 ml. of 70% (v./v.) ethanol, in 100 × 16 mm. test tubes tightly sealed with rubber stoppers, are heated in a 60°C. water bath for 90 min. with constant shaking. Tightly fitting rubber stoppers are essential. Even a temporarily "popped" stopper produces a high zein value. *Without precooling*, or unstoppering, the tubes are shaken vigorously on a vortex-type mixer and centrifuged 3 min. at top speed (approximately 1,600 × g) in a bench centrifuge. The tubes are again shaken on a vortex mixer and recentrifuged for 5 min. Using 0.1-ml. serological pipets, 0.1-ml. samples of supernatant solution are transferred to 23 mm. diameter discs of Whatman 3MM filter paper. Each filter paper disc is supported halfway up a straight stainless-steel pin pushed through the center of the disc and into a cork base. The pins serve as handles and also keep the discs separated during the washing procedures. After absorption, but before complete drying, each disc is placed in 5% sodium chloride solution. All discs, including controls, are bulked in the same beaker of saline. At least 1 hr. after the last disc has been immersed, the following bulk washing procedure is applied (temperature is ambient unless specifically noted otherwise): 1) sodium chloride solution, 5%, 15 min., twice; 2) distilled water, 15 min., once; 3) anhydrous ethyl ether, 10 min., once; 4) cold (-20°C.) acetone, 10 min., once; 5) room temperature acetone, 10 min., once; and 6) anhydrous ethyl ether, 10 min., twice. The discs are finally air-dried and digested for micro-Kjeldahl nitrogen analysis (5). For calculating zein nitrogen per 100 mg. of ground sample, a total supernatant volume of 0.4 ml. (equal to the volume of 70% ethanol added) is assumed.

#### Procedural Notes

1. Vigorous mixing of the sample and 70% ethanol prior to incubation at 60° is *not* recommended since this tends to throw sample up the tube walls.
2. Precooling prior to the first centrifugation results in low readings. For this reason incubations should be staggered so that centrifugations can be performed immediately after the tubes are removed from the water bath.
3. Without the second mixing and recentrifugation the second duplicate from an incubation tube is almost invariably lower than the first, presumably because some layering occurs during the first centrifugation.
4. A convenient system which facilitates washing, changing wash solutions, and draining the discs is to have two beakers, one resting inside the other. The inner beaker has holes, approximately 5 mm. in diameter, in the bottom, and two glass rod handles radiating from the rim. The turbulence created by moving the inner beaker containing the discs, up and down, permits efficient mixing and rapid changing of solutions.
5. In digesting for micro-Kjeldahl analysis it is advantageous to start the digestion at a lower temperature than usual in order to prevent excessive frothing caused by the unusually large amounts of carbon contributed by the disc.

TABLE I. ZEIN CONTENT (mg. per g. whole kernel) OF REPLICATE SAMPLES FROM GROUND PREPARATIONS OF THE NORMAL AND OPAQUE-2 VERSIONS OF TWO MAIZE INBREDS<sup>1</sup>

Replicate No.	W64A				Oh43			
	+							
	1 <sup>2</sup>		2 <sup>2</sup>		1 <sup>2</sup>		2 <sup>2</sup>	
	1 <sup>3</sup>	11 <sup>3</sup>	1 <sup>3</sup>	11 <sup>3</sup>	1 <sup>2</sup>	2 <sup>2</sup>	+	o <sub>2</sub>
1	9.4	9.4	9.8	10.1	3.6	3.7	7.6	4.3
2	10.0	9.7	9.6	9.5	3.8	3.5	7.7	4.4
3	9.6	9.5	9.7	...	3.5	3.5	7.7	4.5
4	9.4	9.5	10.1	...	3.6	3.6	...	4.4

<sup>1</sup>Each replicate value is mean of duplicate discs.

<sup>2</sup>Preparation number

<sup>3</sup>Assay number

TABLE II. ANALYSIS OF VARIANCE OF ZEIN CONTENT IN NORMAL MAIZE INBRED W64A AMONG SEPARATE PREPARATIONS GROUND AT DIFFERENT TIMES, BETWEEN ASSAYS MADE FROM EACH PREPARATION ON DIFFERENT OCCASIONS, AMONG REPLICATES WITHIN ASSAYS AND BETWEEN DISCS WITHIN REPLICATES AND THE EXPECTED MEAN SQUARES

Source	Debrees of Freedom	Mean Square	Expected Mean Square
Among preparations	2	0.612755	$\sigma_d^2 + 2\sigma_r^2 + 8.70286\sigma_a^2 + 16.48\sigma_p^2$
Between assays within preparations	3	0.611248	$\sigma_d^2 + 2\sigma_r^2 + 7.90476\sigma_a^2$
Among replicates within assays	19	0.245307	$\sigma_d^2 + 2\sigma_r^2$
Between discs within replicates	25	0.141342	$\sigma_d^2$

## RESULTS AND DISCUSSION

The reproducibility of the method is good, as illustrated in Table I for the normal and *opaque-2* versions of two maize inbreds. More extensive laboratory analyses of preparations of corn samples ground at different times and assayed on several occasions using varying numbers of replicates per assay and duplicate discs for each replicate were also performed for normal maize inbred W64A. Analysis of variance of the results and the expected mean squares are given in Table II (6). To estimate the inherent variability of each source of variation, the mean squares were equated to the expected mean squares to solve for the estimated variance components:

Among preparations	$\hat{\sigma}_p^2 = -0.00215$
Between assays within preparations	$\hat{\sigma}_a^2 = 0.0463$
Among replicates within assays	$\hat{\sigma}_r^2 = 0.0520$
Between discs within replicates	$\hat{\sigma}_d^2 = 0.1413$

From these variance components it may be seen that the greatest single source of error is that between discs. These values permit the standard error to be calculated for a variety of experimental circumstances. For example, the standard error for a single preparation, a single assay, one replicate ( $r = 1$ ), and

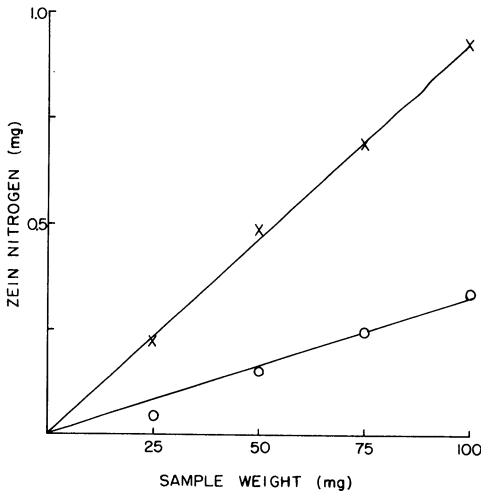


Fig. 1. Relationship between initial sample weight and zein nitrogen recovered. x = W64A+; o = W64A (opaque-2).

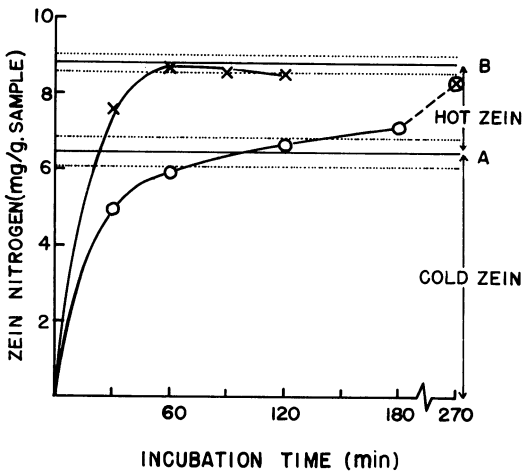


Fig. 2. Progress curve for extraction of zein from ground whole kernels at 25° (o), 60° (x), and 25°C. followed by 60°C. (x) (see text) by the present method. "Hot zein" and "cold zein" refer to the zein fractions of Baudet et al. (4) determined on 10 replicate samples of the same material to provide a standard for comparison; horizontal lines refer to mean ± standard deviation of individual replicates; A for cold zein, B for total (hot + cold) zein.

one disc (d = 1) per replicate is

$$\hat{\sigma} = \sqrt{\hat{\sigma}_p^2 + \hat{\sigma}_a^2 + \frac{\hat{\sigma}_r^2}{r} + \frac{\hat{\sigma}_d^2}{d}} = 0.489$$

For this purpose the negative variance component for preparations was assumed to be equal to zero. Thus, for a determination based on a single disc the coefficient of variation, or ratio (percent) between the standard error and the

mean, is only 5.2%, and for duplicate discs ( $r = 1$ ,  $d = 2$ ) is 4.3%. In practice it is most convenient, and deemed safest, to assay a single disc from each of the two replicates ( $r = 2$ ,  $d = 1$ ), in which case the coefficient of variation is 4.0%. If two replicates and two discs per replicate ( $r = 2$ ,  $d = 2$ ) were used, the coefficient of variation would be 3.5%.

There is a linear relationship between the zein nitrogen recovered and the weight of sample extracted as illustrated in Fig. 1 using the normal inbred W64A and its isogenic *opaque-2* counterpart. This makes it possible to work with samples smaller than 100 mg. Thus, single kernel analyses are possible.

The time course for extractions of zein at 25° and 60° C. (Fig. 2) provides the rationale for selecting a 90-min. extraction at 60° C. for the present assay. The 60° extraction was complete in 60 min. and the amount of zein extracted is in excellent agreement with the mean value for the "total" zein obtained by analyzing 10 replicate samples of the same ground material by the method of Baudet et al. (4) (Fig. 2). Prolonging the extraction may result in a gradual loss of extractability at 60° C. At 25° C. an increase in extracted zein was still evident after 180 min., which would appear to preclude a reliable determination of "cold" zein within the desirably short time period examined. A further 90 min. at 60° C. then failed to yield all the zein, again suggesting a loss of extractability by prolonged treatment. The coefficients of variation for the zein obtained by applying the cold and hot extractions of Baudet et al. (4) to the 10 replicate samples mentioned above were 5.9 and 9.2%, respectively. The coefficient of variation for the "total" zein, i.e. hot plus cold, is only 2.5%, suggesting that each set of hot and cold extracts are complementary, and that there may be no real qualitative difference between hot and cold zein. This suggestion is reinforced by the fact that Baudet et al. (4) demonstrated closely similar amino acid compositions for the two fractions.

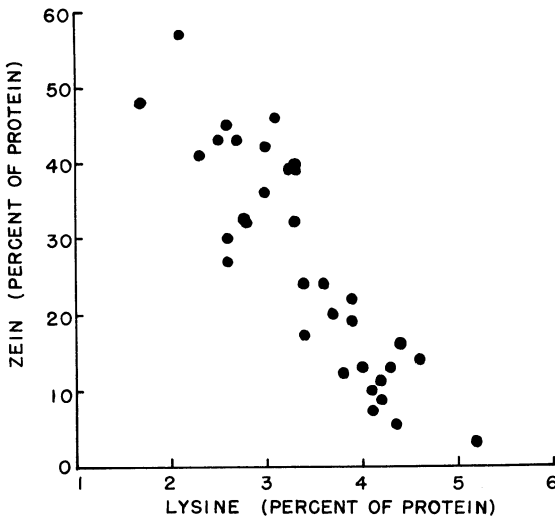


Fig. 3. Relationship between zein and lysine content in mature samples of normal, high-lysine, and starch-modified lines of maize.

Losses of zein in the washing procedure are minimal. Using laboratory zein losses amount to less than 3%. The minimum "critical peptization temperature" for binary mixtures of acetone and water is slightly above  $-10^{\circ}\text{C}$ . (7). When only a few discs are being washed it is possible to progress directly from water (step 2) to cold acetone (step 4) without appreciable losses of zein, if a relatively large volume of cold acetone is used and mixing is rapid. However, with larger numbers of discs the water retained, even after prolonged draining, is sufficient to provide zein-solubilizing conditions in the discs at the time they are plunged into the acetone. Anhydrous ether at room temperature (step 3) overcomes this problem by removing much of the water before cold acetone treatment.

The method has proved of value in following the pattern of zein accumulation during endosperm development in *opaque-2* and normal maize (8) and other mutants producing altered protein compositions in the endosperm (A. Dalby and C. Y. Tsai, in preparation).

A reciprocal relationship has been shown between the quantities of zein and lysine in maize seed (3). Figure 3 illustrates the use of the present method of zein determination in demonstrating this reciprocity with a number of normal, high-lysine, and starch-modified lines. The method may be of value, therefore, as an additional tool in the search for maize selections having a high percentage of lysine in the total seed protein as the result of a low zein content in the endosperm.

Filter paper discs have been used hitherto in radioisotope incorporation experiments in nucleic acid (9) and protein synthesis (10) research as a means of washing large numbers of samples to free them from unincorporated labelled compounds. The present application of the filter paper disc technique is possible because of the unique solubility properties of the prolamine, zein, which permit a variety of solvents to be employed to remove both non-nitrogenous and nitrogenous non-zein impurities. Thus the method is direct, requiring no preliminary delipidation, and is based on nitrogen content; rapid, providing a means of processing large numbers of samples simultaneously; reproducible; and economical in supplies, equipment, and labor. Finally, the method should be readily applicable to the determination of the prolamine fraction in other cereal grains.

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